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Carbohydrate and Peptide Structure of the α - and β -Subunits of Human Chorionic Gonadotropin from Normal and Aberrant Pregnancy and Choriocarcinoma

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Human chorionic gonadotropin (hCG), purified from the urine of 14 individuals with normal pregnancy, diabetic pregnancy, hydatidiform mole, or choriocarcinoma, plus two hCG standard preparations, was examined for concurrent peptide-sequence and asparagine (N)- and serine (O)-linked carbohydrate heterogeneity. Protein-sequence analysis was used to measure amino-terminal heterogeneity and the "nicking" of internal peptide bonds. The use of high-pH anion-exchange chromatography coupled with the increased sensitivity of pulsed amperometric detection (HPAE/PAD) revealed that distinct proportions of both hCG α - and β -subunits from normal and aberrant pregnancy are hyperglycosylated, and that it is the extent of the specific subunit hyperglycosylation that significantly increases in malignant disease.

Peptide-bond nicking was restricted to a single linkage (β 47-48) in normal and diabetic pregnancy, but occurred at two sites in standard preparations, at three sites in hydatidiform mole, and at three sites in choriocarcinoma β -subunit. In the carbohydrate moiety, α -subunit from normal pregnancy hCG contained nonfucosylated, mono- and biantennary N-linked structures (49.3 and 36.7%, means); fucosylated biantennary and triantennary oligosaccharides were also identified (7.3 and 6.9%). In choriocarcinoma α -subunit, the level of fucosylated biantennary increased, offset by a parallel decrease in the predominant biantennary structure of normal pregnancy ($P < 0.0001$). The β -subunit from normal pregnancy hCG contained fucosylated and nonfucosylated biantennary N-linked structures; however, mono- and triantennary oligosaccharides were also identified (4.6 and 13.7%). For O-linked glycans, in β -subunit from normal pregnancy, disaccharide-core structure predominated, whereas tetrasaccha-

ride-core structure was also detected (15.6%). A trend was demonstrated in β -subunit: the proportions of the nonpredominating N- and O-linked oligosaccharides increased stepwise from normal pregnancy to hydatidiform mole to choriocarcinoma. The increases were: for monoantennary oligosaccharide, 4.6 to 6.8 to 11.2%; for triantennary, 13.7 to 26.7 to 51.5% and, for O-linked tetrasaccharide-core structure, 15.6 to 23.0 to 74.8%. For hCG from individual diabetic pregnancy, the principal N-linked structure (34.7%) was consistent with a biantennary oligosaccharide previously reported only in carcinoma; and sialylation of both N- and O-linked antennae was significantly decreased compared to that of normal pregnancy.

Taken collectively, the distinctive patterns of subunit-specific, predominant oligosaccharides appear to reflect the steric effect of local protein structure during glycosylation processes. The evidence of alternative or "hyperbranched" glycoforms on both α - and β -subunits, seen at low levels in normal pregnancy and at increased or even predominant levels in malignant disease, suggests alternative substrate accessibility for Golgi processing enzymes, α 1,6fucosyltransferase and N-acetylglucosaminyltransferase IV, in distinct proportions of subunit molecules.

Key Words: HCG α - and β -subunits; aberrant pregnancy; diabetic pregnancy; carcinoma; N- and O-linked glycans.

Introduction

Human chorionic gonadotropin (hCG), a glycoprotein hormone, is produced by normal trophoblast cells of the placenta during pregnancy, by hyperplastic cells in hydatidiform mole and malignant cells in choriocarcinoma, and by testicular and other, nontrophoblastic neoplasms. The binding of hCG to plasma membrane receptors in the ovary and testis activates the adenylate cyclase enzyme system,

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Structure of hCG α - and β -Subunits/Elliott et al.

Endocrine

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 α -subunit, molecular weight: 10,207 (peptide) + 3,756 (carbohydrate)

ala-pro-asp-val-gln-asp-cys-pro-glu-cys-thr-leu-gln-glu-asp-pro-phe-phe-ser-gln-pro-gly-ala-pro-ile-leu-
 1 2 \uparrow 3 \uparrow 4 N
 gln-cys-met-gly-cys-cys-phe-ser-arg-ala-tyr-pro-thr-pro-leu-arg-ser-lys-lys-thr-met-leu-val-gln-lys-asn-val
 27 42 \uparrow 43 N 52
 thr-ser-glu-ser-thr-cys-cys-val-ala-lys-ser-tyr-asn-arg-val-thr-val-met-gly-gly-phe-lys-val-glu-asn-
 54 78
 his-thr-ala-cys-his-cys-ser-thr-cys-tyr-tyr-his-lys-ser
 79 92

 β -subunit, molecular weight: 15,532 (peptide) + 8,351 (carbohydrate)

ser-lys-glu-pro-leu-arg-pro-arg-cys-arg-pro-ile-asn-ala-thr-leu-ala-val-glu-lys-glu-gly-cys-pro-val-cys-ile-thr-val-asn-
 1 \uparrow 2 N 13 30
 thr-thr-ile-cys-ala-gly-tyr-cys-pro-thr-met-thr-arg-val-leu-gln-gly-val-leu-pro-ala-leu-pro-gln-val-val-cys-asn-tyr-arg-
 31 43 \uparrow 44 \uparrow 45 47 \uparrow 48
 asp-val-arg-phe-glu-ser-ile-arg-leu-pro-gly-cys-pro-arg-gly-val-asn-pro-val-val-ser-tyr-ala-val-ala-leu-ser-cys-gln-cys-
 61 75 \uparrow 76
 ala-leu-cys-arg-arg-ser-thr-thr-asp-cys-gly-gly-pro-lys-asp-his-pro-leu-thr-cys-asp-asp-pro-arg-phe-gln-asp-ser-ser-ser-
 O O O O
 ser-lys-ala-pro-pro-pro-ser-leu-pro-ser-pro-ser-arg-leu-pro-gly-pro-ser-asp-thr-pro-ile-leu-pro-gln
 121 127 132 138 145

Fig. 1. The primary structure of the α - and β -subunits of hCG with carbohydrate attachment sites (49). Numbers are amino acid sequence order. N indicates asparagine residues with N-linked oligosaccharide, and O indicates serine residues with O-linked glycans. Arrows (T) denote sites of potential amino-terminal heterogeneity and nicking of internal peptide bonds. Molecular weight for α -subunit calculated based on an intact primary sequence, five disulfide bonds, one sialylated monoantennary and one sialylated biantennary N-linked oligosaccharide. Molecular weight for β -subunit calculated based on an intact primary sequence, six disulfide bonds, one sialylated biantennary and one sialylated, fucosylated biantennary N-linked oligosaccharide, and four sialylated, disaccharide-core O-linked oligosaccharides.

promoting steroidogenesis (1,2). During the early weeks of pregnancy, the trophoblast secretion of hCG stimulates ovarian production of progesterone, both hormones being critical to successful implantation and maintenance of the fetal graft. Additional, less-defined properties of hCG include immuno- and invasion-suppressive activities (3,4).

hCG is composed of two dissimilar subunits, designated α and β . The primary sequence of α -subunit consists of 92 amino acids with 5 disulfide bonds and N-linked carbohydrate attachment sites at asparagine residues α 52 and α 78. Biological activity is conferred upon noncovalent association with β -subunit (5). The peptide portion of β -subunit consists of 145 amino acids with 6 disulfide bonds and N-linked carbohydrate attachment sites at asparagine residues β 13 and β 30 (5,6). The most striking difference between the protein sequence of the β -subunit of hCG and those of the other glycoprotein hormones (LH, TSH, FSH) is the inclusion of a 25- to 30-residue carboxy-terminal extension. O-linked carbohydrate attachment sites are located on this serine/proline-rich domain at serine residues β 121, β 127, β 132, and β 138 (Fig. 1).

The carbohydrate processing pathway of hCG resembles

reticulum, precursor oligosaccharide is transferred to two asparaginyl residues of each nascent α - and β -peptide chain (7,8); as the combined $\alpha\beta$ dimer is transported through the Golgi, glycosidase and glycosyltransferase activities complete the processing of N-linked oligosaccharide and the addition of O-linked oligosaccharide to the β carboxy-terminal extension (9-11). The processes that lead to this heterodimer with final glycosylation at eight sites are complex, involving the critical folding of β -subunit both before and after combination with α -subunit (12). The recent determination of the crystal structure of hCG indicates a cystine-knot motif similar to the core regions of platelet-derived and transforming growth factors (6). Since hCG is produced in normal, hyperplastic, and malignant cells, it is a potential marker of cellular change and, as such, is of particular interest as a model for investigating the processes of a normal invasive state (pregnancy) vs those of a malignant condition.

Peptide heterogeneity has been reported at the amino-terminus of α -subunit and, in β -subunit, peptide-bond cleavages ("nicking") have been identified (13-17). In the carbohydrate moiety, numerous permutations have been reported in both the N- and the O-linked glycans of hCG

from normal pregnancy (18–24).^{*} In hCG from choriocarcinoma, unusual N-linked biantennary and triantennary glycans (25) with extreme variation in the sialic acid content (26) and increased O-linked tetrasaccharide-core structure have been reported (23, 24). Progressive changes in the glycosylation content of the hCG produced as pregnancy advances have also been described (27–29). The carbohydrate moiety has been shown to be essential for the biological activity of hCG; complete or partial removal of the N-linked oligosaccharides abolishes steroidogenesis, although the precise nature of the structural involvement remains uncertain (30).

Previous structural studies of hCG that focused on carbohydrate heterogeneity or on peptide integrity have been limited to a few individual samples (purified and analyzed by differing methodologies), to CR standard preparations of hCG, or to subunit preparations purified from proprietary crude extracts of pooled pregnancy urine. The assessment of inherent heterogeneity is difficult when both varied results and differing protocols are reported, while the isolation of hCG from commercial extracts raises concerns regarding the inclusion of urine from abnormal pregnancies, and the preferential selection or deletion of certain forms of heterogeneity in the initial, unknown stages of purification. In addition, any investigation of hCG purified from pooled samples cannot address the issue of heterogeneity, which is attributable to individual variation. The purpose of this study was to describe more thoroughly the combined peptide and carbohydrate heterogeneity of hCG. To this aim, we sought:

1. To analyze an increased number of samples from individuals to assess that level of heterogeneity owing solely to individual variation;
2. To compare directly hCG from normal and aberrant states of pregnancy and from choriocarcinoma, all purified in the same manner;
3. To separate hCG into its respective α - and β -subunits for improved resolution of data;
4. To examine both the peptide-sequence integrity and the accompanying N- and O-linked carbohydrate structures; and
5. To address any concurrent peptide-carbohydrate relationship.

Results

Purification of hCG α - and β -Subunits

hCG was isolated from 14 separate urine collections obtained from individuals with normal pregnancy, diabetic pregnancy,^{**} hydatidiform mole, or choriocarcinoma and

then tested for purity as described in Materials and Methods. In addition, two standard preparations (CR127 and CR129 hCG) were investigated. Following the dissociation and resolution of the hCG into its constituent α - and β -subunits, the integrity of the respective subunit pools was assessed for nondissociated hCG and for the alternative subunit using hCG- and subunit-specific immunoassays. The determined mean values were as follows: for the α -pools, 0.4% hCG- and 2.7% β -immunoreactivity, and for the β -pools, 2.2% hCG- and 1.8% α -immunoreactivity. Amino-terminal sequence analysis revealed only α -subunit sequences in the reduced and S-carboxymethylated (RCM) α -preparations and only β -subunit sequences in the RCM β -subunit preparations.

Determination of Amino-Terminal Heterogeneity and Peptide-Bond Nicking

In an earlier study by this laboratory, heterogeneity was detected at the amino-terminus of α -subunit at $\alpha 3$ and $\alpha 4$, but solely in hydatidiform mole and choriocarcinoma hCG (14). Internal peptide-bond nicking was identified at two sites: $\beta 44$ –45 and $\beta 47$ –48. The earlier study was limited by the inherent difficulty in resolving overlying α - and β -subunit sequences in the nondissociated hCG preparations, plus the presence of additional amino-terminal sequences if either or both subunits were internally nicked. In this study, 16 purified hCG samples were separated by dissociation and size exclusion into their respective α - and β -subunits, and then reduced and S-carboxymethylated prior to amino-terminal sequence analysis. This approach allowed greater resolution of peptide heterogeneity specific to the individual subunits and resulted in the detection of additional nicking sites. Sequence analysis revealed that 14 of 15 RCM α -subunits exhibited heterogeneity at the amino-terminus, with 6–18% of the molecules commencing at $\alpha 3$ and/or $\alpha 4$ (Table 1). The extent of amino-terminal heterogeneity for α -subunit was similar in all categories examined: for the four individual and two CR standard preparations from normal pregnancy (mean: 9.4 and 11.1%, respectively), for diabetic pregnancy (sample D1, 6.5%), for the three hydatidiform mole (7.9%), and for the four choriocarcinoma (12.6%) individual samples. For β -subunit, only 1 of 15 samples, choriocarcinoma C7, exhibited any amino-terminal heterogeneity (40.5% commencing at $\beta 2$).

Internal peptide-bond nicking was detected in 2 of 15 α -subunits (CR129 standard preparation and choriocarcinoma C2). Both were nicked at a previously unreported site, between $\alpha 42$ –43 (4 and 19%, respectively) (Table 1). In β -subunit, internal nicking was detected in 12 of 15 subunits tested. As in the earlier study (14), cleavage

^{*}Complex-type asparagine (N)-linked oligosaccharide structure is classified according to the number of antennae or branches attached to the Man₅GlcNAc₂ core (e.g., mono-, bi-, tri-, tetra-antennary) and is designated herein according to the sugar present at the desialylated nonreducing terminus using the following code: G, Galactose; M, Mannose; A, N-acetylglucosamine. The presence of core fucose (attached to the GlcNAc linked to asparagine) is designated by F. Serine (O)-linked oligosaccharides are classified according to the number of neutral sugar residues (e.g., di-, tetra-saccharide).

Table 1
Amino-Terminal Heterogeneity and Internal Peptide-Bond Nicking in Separated RCM α - and β -Subunits

Sample code	Amino-terminal sequences detected in α starting at ^a					Amino-terminal sequences detected in β starting at ^a						Total ^b Total ^c	Total ^c Total ^c
	$\alpha 1$	$\alpha 3$	$\alpha 4$	Total ^b $\alpha 3, 4$	$\alpha 43$	$\beta 1$	$\beta 2$	$\beta 44$	$\beta 45$	$\beta 48$	$\beta 76$	$\beta 44, 45, 76$	$\beta 44-76$
Normal pregnancy													
Standard CR127	1.0	0.02	0.12	0.14	0	1.0	0	0	0.07	0.12	0	0.07	0.19
Standard CR129	1.0	0	0.11	0.11	0.04	1.0	0	0	0.04	0	0	0.04	0.04
Individual P3	1.0	0	0.08	0.08	0	1.0	0	0	0	1.04	0	0	1.04
Individual P7	1.0	0.22	0	0.22	0	nd ^d	nd	nd	nd	nd	nd	nd	nd
Individual P8	1.0	0	0.07	0.07	0	1.0	0	0	0	0	0	0	0
Individual P9	1.0	0	0.06	0.06	0	1.0	0	0	0	0	0	0	0
Diabetic pregnancy													
Individual D1	1.0	0.07	0	0.07	0	1.0	0	0	0	0	0	0	0
Individual D2	1.0	0	0	0	0	1.0	0	0	0	0.08	0	0	0.08
Hydatidiform mole													
Individual M1	1.0	0.06	0	0.06	0	1.0	0	0.09	0	0.09	0.06	0.15	0.24
Individual M2	1.0	0.05	0.07	0.12	0	1.0	0	0.04	0	0.03	0.08	0.12	0.15
Individual M4	1.0	0	0.08	0.08	0	1.0	0	0	0	0.98	0	0	0.98
Choriocarcinoma													
Individual C1	1.0	0.06	0.12	0.18	0	1.0	0	0.12	0.17	0.08	0	0.29	0.37
Individual C2	1.0	0	0.15	0.15	0.19	1.0	0	0	0.27	0.16	0	0.27	0.43
Individual C3	1.0	0.09	0	0.09	0	1.0	0	0.05	0.07	0.13	0	0.12	0.25
Individual C5	1.0	0.03	0.13	0.16	0	1.0	0	0	0	1.06	0	0	1.06
Individual C7	nd	nd	nd	nd	nd	1.0	0.68	0	0.03	0	0	0.03	0.03

^aSequences were determined from a minimum of eight cycles of analysis. Finding a sequence required identification in at least four of the first six consecutive residues with comparable levels (within 50%). Finding at least two unique residues (those not in any other sequence) was also a requirement. Concentrations of at least two unique residues were used to estimate amounts of sequences. These were Val 4 and Pro 2 for $\alpha 1$, Val 2 and Gln 3 for $\alpha 3$, Gln 2 and Pro 5 for $\alpha 4$, and Lys 2 and Lys 3 for $\alpha 43$. The unique residues were Pro 4 and Lys 2 for $\beta 1$, Leu 4 and Arg 5 for $\beta 2$, Gln 3 and Gly 4 for $\beta 44$, Gln 2 and Gly 3 for $\beta 45$, Ala 4 and Gln 6 for $\beta 48$, and Asn 2 and Val 5 for $\beta 76$. Molar values were normalized to the concentration of sequence starting at residue 1.

^bTotal amino-terminal heterogeneity = sum of all normalized sequences that do not begin at residue 1 (for α -subunit, $\alpha 3$ and/or $\alpha 4$; for β -subunit, $\beta 2$). Percent amino-terminal heterogeneity = total amino-terminal heterogeneity divided by total normalized sequences $\times 100$. Example: P7 α amino-terminal heterogeneity = 0.22 divided by 1.22 (1.00 plus 0.22) $\times 100 = 18\%$.

^cTotal internal peptide-bond nicking = sum of all normalized sequences that begin at residue $\alpha 43$ for α -subunit; sum of all normalized sequences that begin at residues $\beta 44$, $\beta 45$, $\beta 48$, and $\beta 76$ for β -subunit. Percent peptide bond nicked at a particular site = normalized value for nick site $\times 100$.

^dnd = not determined.

between residues $\beta 47$ -48 was the site of greatest potential for nicking and was detected in all categories. Nicking at $\beta 47$ -48 varied from 0-1.06, but tended to group at the extremes of the range. Nicking was also identified between residues $\beta 44$ -45, and at two new sites, $\beta 43$ -44 and $\beta 75$ -76. With the exception of 7 and 4% nicking at $\beta 44$ -45 in CR127 and CR129 standard preparations, internal nicking at sites other than $\beta 47$ -48 was confined to hydatidiform mole and choriocarcinoma. In hydatidiform mole, β -subunit was nicked at $\beta 47$ -48, and at two new sites: $\beta 43$ -44 and $\beta 75$ -76. Choriocarcinoma β -subunit was nicked at $\beta 47$ -48 as in pregnancy, at $\beta 44$ -45 as in CR standard preparations, and at $\beta 43$ -44 as in hydatidiform mole.

The finding of β -subunit sequences starting at $\beta 44$, $\beta 45$, and $\beta 48$ shows the presence of extra amino termini but

does not prove that prior residues ($\beta 43$, $\beta 44$, and $\beta 47$) are present. Carboxypeptidase Y carboxyl-terminal sequence analysis was used to address this issue. β -subunit preparations M4 and C5 are near-equal mixtures of peptides starting at $\beta 1$ and $\beta 48$ (Table 1). This was interpreted as the occurrence of 100% nicking between $\beta 47$ -48. Carboxypeptidase Y released Gly, Gln, Pro, and Leu in the approximate ratio of 1:2:1:2 from both M4 and C5 β -subunit preparations (not shown). This is consistent with the release of three carboxy-terminal amino acids (Gln 145, Pro 144, and Leu 143) from peptide $\beta 48$ -145 and three carboxy-terminal amino acids (Gly 47, Gln 46, and Leu 45) from peptide $\beta 1$ -47. The collective data from the amino- and carboxy-terminal sequence analyses are consistent with little if any loss of amino acids from peptides $\beta 1$ -47 or

Table 2
Asparagine-Linked Oligosaccharides of RCM hCG α -Subunit

Sample code	GM, %	GGF, %	GGM, %	GG, %	GGGF, %	GGG, %	Sialic acid, pmol/pmol oligosaccharide	% ^a
Normal pregnancy								
Standard CR127	48.0	8.9 ^b	0	38.4 ^b	0 ^c	4.8 ^c	1.50	95.6 ^d
Standard CR129	49.7	3.1	0	37.8	0	9.4	1.47	91.9
Individual P3	53.8	7.3	0	36.4	2.6	0	1.76	117.9
Individual P7	40.7	7.9	0	37.1	14.3	0	1.70	98.2
Individual P8	41.5	7.3	0	41.1	10.1	0	1.73	102.8
Individual P9	61.9	9.4	0	29.2	0	0	1.44	103.4
Diabetic pregnancy								
Individual D1	12.0	7.0	34.8	24.2	22.0	0	0.71	33.7
Individual D2	28.3	2.8	34.0	17.1	17.7	0	0.93	49.2
Hydatidiform mole								
Individual M1	43.4	16.7	0	32.3	3.6	3.9	1.32	80.3 ^d
Individual M2	53.1	4.4	0	36.6	0	5.9	1.38	90.0
Individual M4	35.7	0	nd ^e	54.6	0.5	9.2	1.39	79.9
Choriocarcinoma								
Individual C1	38.5	16.0 ^b	3.6	18.9 ^b	6.2	16.7	1.70	92.2 ^d
Individual C2	56.6	24.2	3.7	5.7	0	9.8	1.36	88.9
Individual C3	71.1	19.1	2.2	4.9	0	2.9	1.06	80.3
Individual C5	74.8	18.2	2.3	3.5	0	1.2	0.92	73.1
Individual C7	56.5	20.3	5.1	6.8	8.0	4.3	0.98	61.9

^aPercent = pmol sialic acid/(pmol GM) + 2 (pmol GG, GGM, and GGF) + 3 (pmol GGG and GGGF).

^bBiantennary oligosaccharides contain additional fucose in hCG α -subunit from choriocarcinoma. GGF levels are raised in choriocarcinoma vs normal pregnancy (*t*-test: $P < 0.0001$) and in choriocarcinoma vs hydatidiform mole plus normal pregnancy (*t*-test: $P < 0.0001$). GG levels are lowered in the same comparisons ($P < 0.0001$ and $P < 0.0001$, respectively).

^cIn *t*-tests, no significant difference in proportion of triantennary oligosaccharides (GGGF plus GGG) in hCG α -subunit between normal pregnancy vs choriocarcinoma or in normal pregnancy plus hydatidiform mole vs choriocarcinoma ($P > 0.05$).

^dIn hCG α -subunit, sialic acid content is reduced in choriocarcinoma vs normal pregnancy (*t*-test: $P < 0.01$) and in hydatidiform mole vs normal pregnancy (*t*-test: $P < 0.01$).

^end = not determined.

β 48-145 and with the single cleavage of peptide linkage between β 47-48. Choriocarcinoma sample C2 has peptides starting at β 45 (27%) and β 48 (16%), indicating nicks between β 44-45 and β 47-48 (Table 1). The possibility was considered that the three intervening amino acids (β 45, 46, and 47) could be missing in a portion of molecules. If this were true, up to 43% (27 plus 16%) of the molecules could terminate at Val 44 and up to 16% could be missing β 45-47. Carboxypeptidase Y released Gly, Gln, Pro, Leu, and Val from RCM β -subunit C2 in the approximate ratio of 0.04:1:1:1:0.4 (not shown). This was consistent with the release of Gln 145, Pro 144, and Leu 143 from the carboxy-terminus of the distal peptide (β 48-145), and with the release of Gly 47 at 4% and Val 44 at 40% of the carboxy-terminus of the proximal peptides (β 1 \rightarrow β x). This was consistent with the absence of residues β 45, 46, and 47 in 12% (16 minus 4%) of the molecules.

Oligosaccharide Structure in hCG α - and β -Subunits from Normal Pregnancy

α -Subunit from normal pregnant individuals and from standard preparations was characterized by a predominant

pair of N-linked oligosaccharides: fucose-free mono-antennary (GM: $49.3 \pm 7.9\%$, mean \pm SD) and fucose-free biantennary (GG: $36.7 \pm 4.0\%$) (Table 2). In addition, low but distinct levels of more highly branched structures, fucosylated biantennary (GGF: $7.3 \pm 2.2\%$) and triantennary oligosaccharides (GGG plus GGGF: $6.9 \pm 5.3\%$), were also identified. The oligosaccharides of α -subunit from normal pregnancy contained $101 \pm 9.1\%$ sialylated antennae.

β -Subunit from normal pregnancy hCG contained a predominant pair of biantennary N-linked oligosaccharides: GGF ($50.8 \pm 8.0\%$) and GG ($30.9 \pm 6.6\%$) (Table 3). In addition, distinct levels of monoantennary (GM: $4.6 \pm 2.7\%$) and triantennary (GGG plus GGGF: $13.7 \pm 5.9\%$) oligosaccharides were identified. The sialylation of the N-linked oligosaccharides was near complete at $98.2 \pm 5.5\%$ sialylated antennae. The O-linked oligosaccharide of β -subunit was predominantly disaccharide-core structure ($84.5 \pm 2.8\%$), but a significant level ($15.6 \pm 2.8\%$) of more highly branched tetrasaccharide-core structure was also detected (Table 4). The extent of sialylation was not complete in the O-linked glycans, at $62.9 \pm 13.7\%$ sialylated antennae.